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Preliminary Evidence of Inositol Supplementation Effect on Cell Growth, Viability and Plasma Membrane Fluidity of the Yeast *Saccharomyces cerevisiae*

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Abstract

Inositol is known to initiate positive effects on yeast fermentation performance, cell growth and tolerance against environmental stresses, especially high ethanol concentration. The precise mechanisms by which inositol improves such parameters are yet to be elucidated. The present study was performed to investigate the effect of inositol supplementation on growth, fermentation performance and plasma membrane fluidity during normal gravity fermentation. Yeast cells were grown in a chemically defined fermentation medium with 15% (w/v) glucose, lacking inositol and with 0.1 or 0.4 g/L inositol supplementation. Cell density, cell viability, glucose consumption and ethanol production were monitored for 96 hours. Plasma membrane fluidity was monitored at 24 hours fermentation, representing the respiro-fermentative growth phase, by measuring generalized polarization (GP) of laurdan. The effect of ethanol on membrane fluidity also monitored by measuring GP after exposing cell to 18% (v/v) ethanol. The results of the present experiment indicated that although inositol supplementation did not seem to improve fermentation performance as assessed by glucose consumption and ethanol production, it did improve cell growth leading to higher cell densities. While inositol-supplemented cells had higher growth rates and cell density, they had significantly lower viability, thus the viable cell counts were similar with and without supplementation. There is also evidence that inositol supplementation leads to increased membrane fluidity with significantly lower GP values for yeast cells grown in the inositol supplemented media. However when exposed to high ethanol concentrations, inositol-supplemented yeasts showed a greater GP decrease than those grown without inositol. Thus, interestingly, the non-supplemented yeasts with lower baseline membrane fluidity seemed to better withstand the fluidizing effects of ethanol. We are in the process of confirming the viability of ethanol-treated cells as well as furthering the investigations on inositol effects on stress tolerance and other physiological parameters.

Keywords: inositol, membrane fluidity; generalized polarization; fluorescence spectroscopy; ethanol fermentation

1. Introduction

Depletion of fossil fuels has driven the development of various biofuels, with bioethanol being one of the most widely used around the world[1]. One of the problems in bioethanol production is low ethanol yield due to stuck fermentation. Supplementation of media with various agents has been found to improve yeast performance in fermenting sugar, and consequently to increase ethanol yield. Such agents include yeast extract, catechin, dry spent yeast, glycerol, metal ions (Zn^{2+}, Mg^{2+}, Mn^{2+}), and inositol[2-3].

Myo-inositol (referred to simply as inositol) has been used as a supplement in fermentation media, and found to have positive effects, increasing ethanol tolerance and ethanol productivity[2,4-5], however excess inositol may lead to negative effects. It was proposed that increased availability of inositol leads to altered phospholipid composition of the yeast plasma membrane, which in turn leads to increased stress tolerance[4,6].

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The fluidity of the plasma membrane is influenced by phospholipid composition[7], and therefore it is important to investigate the change of membrane fluidity as related to the phospholipid composition. However, while phospholipid composition is a dominant factor in determining membrane fluidity, numerous other factors also make major contributions to the fluidity[8]. Many studies have inferred membrane fluidity indirectly by determining only the fatty acid unsaturation index[7], while only few have included direct measurement of membrane fluidity. In a recent study we investigated effects of inositol supplementation on yeast ethanol tolerance and membrane fluidity, although lipid compositional analysis was beyond the scope of the study and findings were inconclusive at the range of inositol concentrations studied[9].

Therefore in the present study, we investigated inositol effects on yeast membrane fluidity by direct measurement using the laurdan generalized polarization method, and related the findings to data on fermentation and growth performance.

2. Materials and Methods

2.1 Yeast strains and maintenance

The yeast strain used in this study was A15 (ATCC 38554, originally isolated from canned cherries), selected from a group of strains under study on the basis of known characteristics as promising candidates for use in ethanol production and also on the basis of a range of ethanol and osmotic tolerances[10-11]. Preliminary experiments also showed that this strain has interesting properties, having the highest glucose consumption even though it had the lowest cell viability. Therefore, this strain seems to be a good candidate for improvement of ethanol production by inositol supplementation.Yeasts were maintained on slopes of a complete medium, yeast extract peptone (YEP), containing (w/v) 0.5% yeast extract, 0.5% bacteriological peptone, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 1% glucose and 1.5% agar. Slopes were stored at 4°C and sub-cultured every 6 months. Master cultures were stored in a Sanyo –80°C freezer.

2.2 Growth media and culture conditions

Cells were cultured in the yeast nitrogen base (YNB) medium broth containing 0.69% (w/v) YNB without amino acids and inositol (For Medium, Norfolk/UK) and 0.005% (w/v) amino acid mixture (Sunrise Science, San Diego/USA). Starter cultures were inoculated from slopes and grown overnight (~16 h) at 30°C and 180 opm in an orbital shaker (Paton). For inositol addition experiments, inositol was added to the experimental culture at a final concentration of 0, 0.1, or 0.4 g/L at a time designated as 0 h. We choose this level of inositol based on our survey across the range of previously published studies, of which most concluded that 0.1 g/L inositol showed the best positive effect while excess supplementation led to negative effects. However, we decided to assess a wider range of inositol concentrations in these follow up experiments, to elucidate the effects of inositol more comprehensively.

2.3 Experimental batch culture conditions and sampling

Aerobic cultures were prepared by aseptically inoculating the growth medium (0.69% (w/v) YNB, 0.005% (w/v) amino acid mixture, 15% (w/v) glucose and 0, 0.1 or 0.4 g/L inositol) to give an initial viable cell number of ~10^6 cells/mL, with culturing as previously described[12]. Experimental samples were taken aseptically every 6 hours from 0 to 30 hours and followed at 12 hour intervals until 96 hours. Examination of the samples included measuring growth rate, viable cell numbers, and glucose and ethanol concentrations[12]. Membrane fluidity measurements were performed at 24 hours culture, representing the respiro-fermentative growth phase.

2.4 Growth Rate

Yeast growth was monitored by measuring optical density of the culture at 600 nm (OD_{600nm}) using a Beckman DU 650 spectrophotometer, making dilutions where necessary. Measurements were made using 1 mL (10 mm path length) PMMA cuvettes (Sarstedt).

2.5 Viable Cell Numbers

Viable cell numbers were assessed using the methylene violet staining method and light microscopy (400× magnification) using a Neubauer-type haemo-cytometer. Methylene violet staining is proposed as a better method for monitoring yeast cell viability compared to the traditional methylene blue staining method[13]. An equal volume of the sample was mixed with methylene violet solution (0.01% w/v in 2% sodium citrate solution)[13]. When counting, both live and dead cells were recorded to give the total cells per mL, and the percentage viable cells were calculated.

2.6 Kinetics parameter calculation

Maximum growth rate (μ_{max}) was calculated based on OD values[14] at “exponential” phase such as the time

when the specific growth rate is constant and at its maximum[14]. Glucose consumption rate (*Qs*) was calculated as glucose consumed during the fermentation divided by the fermentation time, while ethanol productivity (*Qp*) was calculated as ethanol concentration produced divided by fermentation time. Ethanol yield (*Yp/s*) was calculated as and expressed as mg ethanol produced per mg glucose consumed[3].

2.7 Determination of membrane fluidity by spectrofluorometric analysis

Membrane fluidity was assessed using steady-state fluorescence spectroscopy, measuring generalized polarization of 6-dodecanoyl-2-dimethylamino-naphthalene (laurdan) following incorporation of the probe into yeast plasma membranes, as outlined by Learmonth[15].

For labelling, an aliquot of washed cells was standardized by diluting with centrifuged (10,000 *g*) supernatant to an $OD_{600\ nm}$ of 0.4 and a volume of 3 mL in a cuvette. Incorporation of the fluorescent probe into yeast cell membranes was accomplished by incubating the standardized washed cell sample with a final concentration of 5 µM laurdan [by adding 6 µL of 2.5 mM laurdan (in ethanol)] for 60 minutes. Samples were incubated at 30°C in the dark with stirring.

In this study, the fluorescent probe laurdan was used to measure generalized polarization, as described by Parasssi et al. (1990)[16] and applied to yeast by Learmonth and Gratton (2002)[8] and Butcher (2008)[17]. After calibrating the PC1 spectrofluorometer (ISS Inc.), the excitation monochromator was set to 340 nm and measurements were taken with emission monochromator wavelengths of 440 and 490 nm, using 8 nm slits for emission and excitation. Generalized polarization measurements were standardized by diluting cells with centrifuged fermentation culture supernatant to an $OD_{600\ nm}$ of 0.4 immediately prior to analysis. A cuvette containing unlabeled cell suspension at the same cell density was used to measure background fluorescence, which was subtracted from the fluorescence readings obtained from the standardized cell suspension.

In a lipid membrane, laurdan exhibits a 50 nm red shift in emission spectrum as the membrane changes from the gel to liquid-crystalline phase. Thus, by measuring the relative emission intensities at wavelengths at the blue and red edges of the spectrum, representing gel (440 nm) and liquid crystalline (490 nm) phases, membrane fluidity may be inferred. The results were expressed as Generalized Polarization (GP) determined using equation 1.

$$GP = \frac{I_{440nm} - I_{490nm}}{I_{440nm} + I_{490nm}} \quad (1)$$

Where I_{440nm} : Emission intensity at 440 nm
I_{490nm} : Emission intensity at 490 nm

2.8 Membrane fluidity after exposure to ethanol

The GP value was monitored as described in the previous sub section over a period of 10 minutes, with measurements taken every minute. After 10 minutes, absolute ethanol was added to the sample cuvette to bring the final concentration of ethanol to 18% (v/v). The GP was then monitored for another 10 minutes. The GP initial drop was calculated as the percentage difference of GP values immediately after ethanol exposure, compared to GP value before ethanol exposure. The GP recovery was determined using equation 2.

$$GP\ Recovery\ (\%) = \frac{GP_{final} - GP_{initial}}{GP_{start} - GP_{initial}} \times 100\% \quad (2)$$

Where:
GP_{final} = Final GP value at 20 minutes
$GP_{initial}$ = GP value immediately after ethanol addition (11^{th} minute)
GP_{start} = The GP values immediately before ethanol addition (10^{th} minutes)

2.9 Determination of glucose

Glucose concentration in fermentation media was determined using the alkaline ferricyanide method of Walker and Harmon[18]. Absorbance of the standards and samples was read at 420 nm with a Beckman DU650 spectrophotometer.

2.10 Determination of ethanol

Ethanol concentration was determined using an enzymatic assay by alcohol dehydrogenase as proposed by Ough and Amerine[19] and modified by Ishmayana et al. (2015)[12] A standard curve of ethanol solution was prepared to give final concentrations of 0.00, 0.01, 0.02, 0.04, 0.05 and 0.06% (v/v). A volume of 25 µL of either standard or sample was added to a reaction tube containing 1.25 mL semicarbazide buffer solution. To the tube,

25 µL NAD^+ and 5 µL alcohol dehydrogenase solution (4000 unit/mL) was added. The mixture was then incubated at 35°C for 40 minutes. After incubation, the absorbance was read at 340 nm using Beckman DU650 spectrophotometer after setting the spectrophotometer to zero with reagent blank. A standard curve was plotted and this was used to calculate the concentration of ethanol in each of the samples.

3. Results and Discussion

3.1 Cell growth and fermentation performance

Growth of yeast cultures is influenced by the composition of both macro- and micro-nutrients in the fermentation media. Inositol, as one of the vitamins, is essential for yeast cell growth and is required in the micro scale. The results of the present study show that inositol improves cell growth and biomass accumulation as indicated by higher $OD_{600\ nm}$ values achieved by yeast cell grown in the presence of inositol (Figure 1). This result confirms previous studies which suggested that inositol affects yeast growth[20-21]. The maximum specific growth rate in respiro-fermentative phase of yeasts grown with inositol was higher than those grown without inositol (Table 1). Interestingly, even though the specific growth rates were higher, the viability was actually substantially lower when cells were grown in the presence of inositol (Figure 2). This led to cultures with and without inositol supplementation having similar viable cell densities, thus likely similar levels of fermentation-active cells. This finding is in contrast with previous published studies which indicated that besides improving cell growth, inositol supplementation also maintained high cell viability during cell growth [22-24]. However, the conditions under which the previous experiments were performed were not exactly the same. Previous studies used lower inositol concentrations (0.01-0.04 g/L) and the glucose concentration also varied from 0.2% w/v [24], 3% w/v[22], to 20% w/v[23]. Therefore, it is difficult to directly compare the present results with previous studies. Furthermore, while increasing the maximum specific growth rate, inositol supplementation led to lower ethanol yield (*Yp/s*) values, seeming to promote greater biomass accumulation but not greater ethanol productivity, in the strain studied. Further exploration on these finding is required to understand the nature of the strain used in the present study, and whether this observation also occurs in other yeast strains.

Table 1. Kinetics parameters of yeasts grown on YNB media without (A), with 0.1 g/L (B) or 0.4 g/L (C) inositol. Data presented is mean of two independent experiments followed by standard error of means (SEM).

Media	μ_{max} (h^{-1})	*Qs* ($mg.mL^{-1}.h^{-1}$)	*Qp* ($mg.mL^{-1}.h^{-1}$)	*Yp/s* ($mg.mg^{-1}$)
A	0.296 ± 0.008	0.787 ± 0.289	0.210 ± 0.061	0.276 ± 0.020
B	0.421 ± 0.019	0.657 ± 0.378	0.129 ± 0.062	0.211 ± 0.022
C	0.430 ± 0.023	0.792 ± 0.303	0.171 ± 0.107	0.192 ± 0.050

Note: μ_{max}= maximum growth rate, *Qs* = glucose consumption rate, *Qp*= ethanol productivity, *Yp/s* = ethanol yield

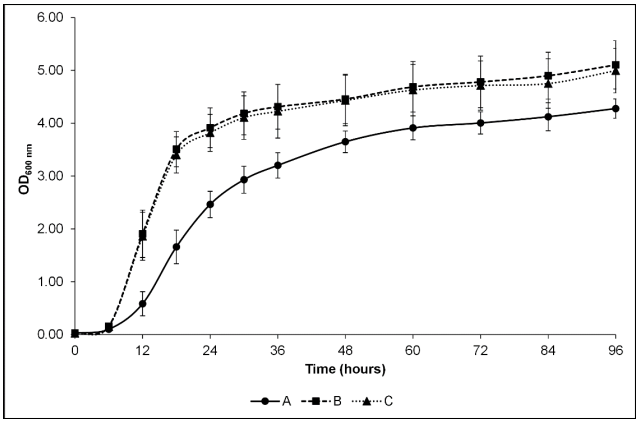

Fig. 1. Growth curve of A15 yeast strain grown on YNB media without (A), with 0.1 g/L (B) or 0.4 g/L (C) inositol supplementation. The initial glucose concentration used in this experiment was 15% (w/v). Data presented are means of two independent experiments and error bars indicate SEM.

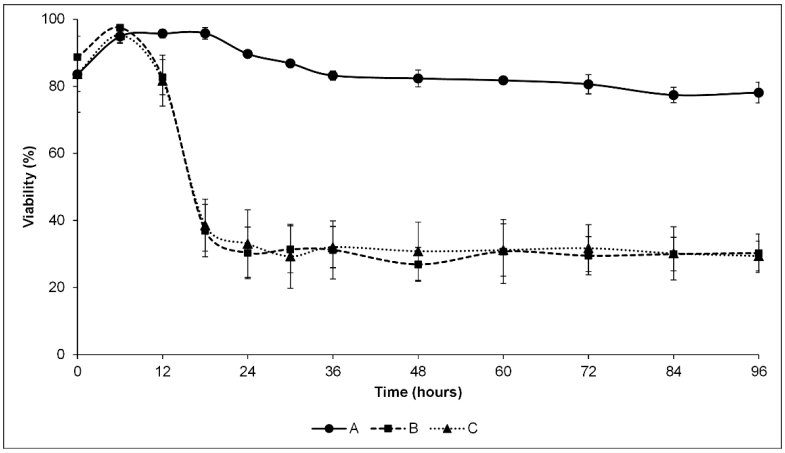

Fig. 2. Viability of A15 yeast strain grown on YNB media without (A), with 0.1 g/L (B) or 0.4 g/L (C) inositol supplementation. The initial glucose concentration used in this experiment was 15% (w/v). Data presented are means of two independent experiments and error bars indicate SEM.

In terms of glucose consumption and ethanol production, there were no substantial differences between cultures grown with or without inositol supplementation, however inositol-supplemented cultures tended to have lower ethanol productivity (Figure 3, Table 1; *Qs*, *Qp* and *Yp/s* values, respectively). This finding is also not in agreement with other previous studies, which indicated that besides improving cell growth, inositol also improved fermentation performance [2,4-5]. Media in which the experiments conducted were different. Caridi (2002)[2] used wine media with 40% (w/v) initial sugar, Nikolić et al. (2009)[5] used immobilized yeast cells with starch hydrolysate while Chi et al. (1999)[4] used synthetic medium with 20% (w/v) sucrose. The present study used synthetic medium but with a relatively high initial glucose concentration [15% (w/v)]. As previously mentioned, nutrition components of the fermentation media are very important factors in determining fermentation performance. Nutrition components other than inositol, in both previous studies and the present study, may also affect the fermentation performance. Caridi (2002)[2] and Nikolić et al. (2009)[5] used complex media, which have rich nutrition, while Chi et al. (1999)[4] and the present study used defined synthetic media which are considered to provide relatively poor nutrition. Even though Chi et al. (1999)[4] and the present study used similar media, different sugars were used (15% (w/v) glucose here, 20% (w/v) sucrose by Chi et al. (2009)[4]. Therefore direct comparison between this and previous studies is problematic. Moreover, a robust statistical analysis could not be performed on the data in the present study, due to technical problems compromising the validity of data collected in a third replicate (not discovered until late in the analysis of the data). Ongoing investigation with more replication is being conducted to allow robust statistical analysis.

The ethanol data in our study indicated that yeast grown without inositol supplementation had better ethanol yields. No precise level has been published at which inositol provides positive effects on fermentation performance. Chi et al. (2009)[4] used 0 and 0.1 g/L inositol in their experiment while Furukawa et al. (2004)[25] used much lower concentrations (10 or 90 μM with no zero level). Ji et al. (2008) who used a different species of yeast and supplementation with 0, 0.01, 0.05, 0.10, 0.15 and 0.20 g/L inositol found that inositol supplementation more than 0.1 g/L led to lowered ethanol productivity. As the present study was a scoping study, utilising a limited range of inositol concentrations, the lowest concentration used in the present study might exceed that promoting positive effects on ethanol productivity. Follow up experiments using a wider range of inositol supplementation are currently being conducted to investigate this phenomenon. Furthermore we are following up with investigation of several yeast strains to discriminate between strain-dependent and generalized yeast responses.

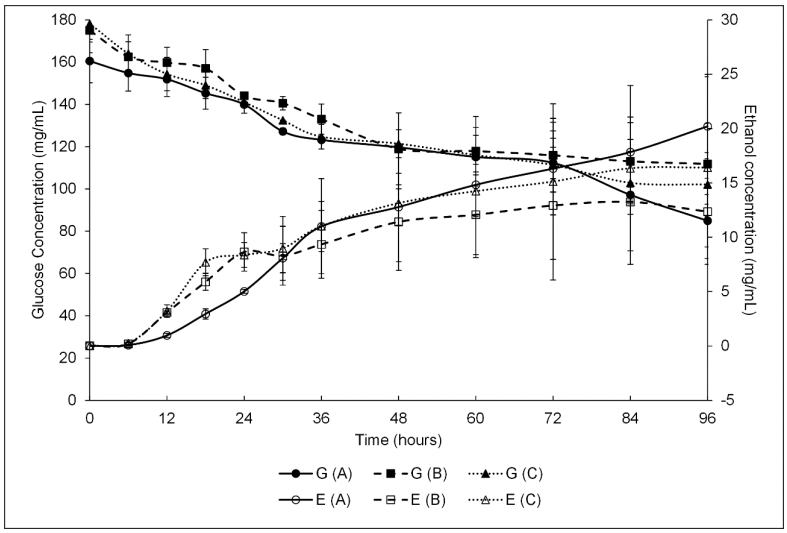

Fig. 3. Glucose consumption and ethanol production of yeast strain A15 in YNB media without (A), with 0.1 g/L (B) and 0.4 g/L (C) inositol supplementation. The letters G and E in the legend indicate glucose or ethanol concentration, respectively. Data are means of two independent experiments and error bars indicate SEM.

3.2 Membrane fluidity

While direct measurement of membrane fluidity of yeast plasma membrane is believed to be more reliable than indirect determination using unsaturation index values, many studies continue to estimate fluidity simply by lipid content. To get a true measure of the fluidity, which can be affected by many factors in addition to lipid unsaturation, in the present study we measured membrane fluidity directly. The results (Figure 4) indicate that yeasts grown in the presence of inositol have substantially lower than generalized polarization values, which indicates that inositol supplementation leads to increased membrane fluidity.

It is most likely that inositol supplementation increases the composition of phosphatidylinositol in the plasma membrane[4], and this may also increase the unsaturation index of the plasma membrane[7], which most likely increases the plasma membrane fluidity. In order to obtain more insight into this phenomenon we will be investigating the plasma membrane composition of yeast grown in the presence and absence of inositol.

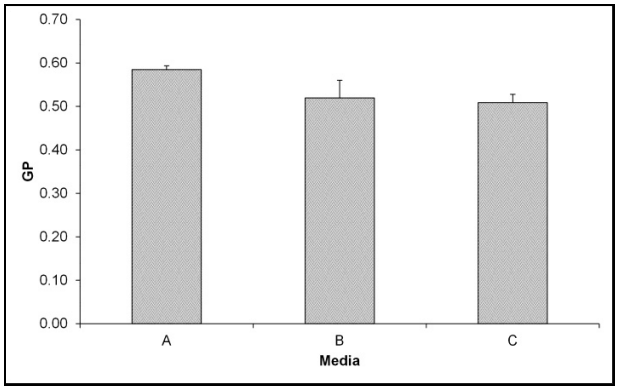

Fig.. 4. Generalized polarization of A15 yeast strain grown in YNB media without (A), with 0.1 g/L (B) and 0.4 g/L (C) inositol supplementation. The initial glucose concentration used in this experiment was 15% (w/v). Data presented are means of two independent experiments and error bars indicate SEM.

3.3 Membrane fluidity change after ethanol exposure

To investigate the effect of inositol supplementation on plasma membrane fluidity change when the yeasts are exposed to high ethanol concentration, we monitored laurdan generalized polarization values before and after exposure to 18% (v/v) ethanol. The results (Figure 5) indicated that prior to ethanol addition yeasts grown without inositol supplementation tended to maintain lower membrane fluidity as indicated by higher GP values.

However after ethanol addition, the increase in fluidity of the non-supplemented cultures was less than that of the inositol supplemented cultures, with the ethanol-affected GP values similar to the "resting" values of the inositol-supplemented cultures. As presented in Table 2, the initial proportional drop of GP values is much higher for cells grown with inositol, but the recovery of GP in these cells was much better than for cells grown without inositol supplementation. This better recovery of membrane fluidity likely indicates better adaption to the toxic effects of ethanol by inositol supplemented cells.

Table 2. Generalized polarization initial drop and recovery (after 10 minutes) after ethanol addition to A15 yeast grown in YNB media without (A), with 0.1 g/L (B) and 0.4 g/L (C) inositol supplementation. The initial glucose concentration used in this experiment was 15% (w/v). Data presented are means of two independent experiments and followed by SEM.

Medium	Initial drop (%)	Recovery (%)
A	14.21 ± 0.74	-7.77± 9.17
B	21.11 ± 1.04	28.40 ± 2.09
C	22.90 ± 0.79	31.02 ± 0.83

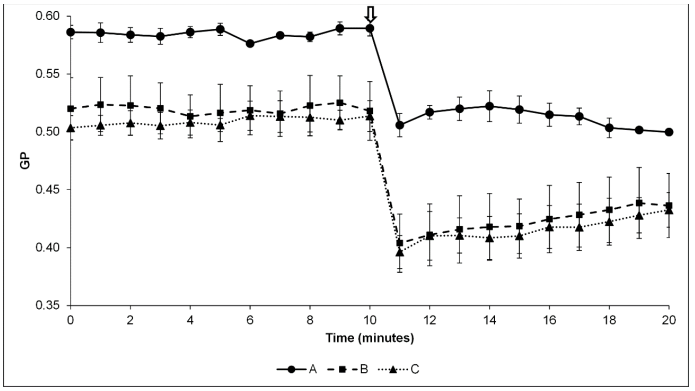

Figure. 5. Changes of yeast strain A15 membrane fluidity as indicated by changes in GP when exposed to 18% (v/v) ethanol. Cells were grown on YNB media without (A), with 0.1 g/L (B) or 0.4 g/L (C) inositol supplementation for 24 hours. The arrow indicates addition of absolute ethanol to give 18% (v/v) final concentration. Data presented are means of two independent experiments and error bars indicate SEM.

This indicates that in the yeast strain studied inositol supplementation increases baseline plasma membrane fluidity and potentiates greater ethanol-mediated lowering of the membrane fluidity. While this may intuitively seem to be undesirable, there is evidence that indicates the phenomenon may contribute to ethanol tolerance. According to Jones and Greenfield[26] elevated membrane fluidity leads to higher plasma membrane permeability. Therefore when exposed to high ethanol concentration, cells with higher membrane fluidity will have higher passive permeability to exclude ethanol from the cell, maintaining lower concentration of intracellular ethanol. However other studies of inositol supplementation suggested that inositol was preventing cell leakage[25] while improving ethanol stress tolerance[4]. The decrease in GP by ethanol was greater in cells grown with inositol supplementation, which may lead to higher passive permeability and eventually better ethanol exclusion. The present study suggests that inositol supplementation actually increases exclusion of intracellular ethanol, but not other cell components. Detailed work to validate this hypothesisis still in progress, including determining whether cell grown in inositol supplemented media have better ethanol stress tolerance.

Conclusion

Inositol supplementation improves cell growth but in contrary it reduces cell viability for the strain used in the present study. Fermentation performance was not markedly affected by inositol in the present experiment, as indicated by no substantial differences glucose consumption, although ethanol production and yield tended to be lower in the presence of inositol. This finding leads us to conclude that better cell viability does not always lead to better cell growth or fermentation performance. However, substantially higher membrane fluidity was observed when the yeasts were grown in the presence of inositol as well as a greater increase in fluidity when exposed to high ethanol concentration. The impacts on ethanol tolerance are being confirmed.

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